表 4　VC および VE が CAL およびその変化量に及ぼす影響

a）全部位

群	ベースライン(n=9,785)	12 W (n=9,785)		24 W (n=9,195)	
	平均　標準偏差	平均　標準偏差		平均　標準偏差	
VC 群	3.41 ± 1.57	3.44 ± 1.45		3.54 ± 1.53	＊＊
VE 群	3.48 ± 1.59	3.31 ± 1.59	＊＊	3.28 ± 1.47	＊＊
VC/VE 群	3.50 ± 1.76	3.46 ± 1.74	＊	3.58 ± 1.79	＊＊
プラセボ群	3.20 ± 1.69	3.19 ± 1.55		3.30 ± 1.55	＊＊

b）ベースライン時が 3 mm 未満の部位

群	ベースライン(n=3,203)	12 W (n=3,203)		24 W (n=3,059)	
	平均　標準偏差	平均　標準偏差		平均　標準偏差	
VC 群	1.76 ± 0.43	2.30 ± 0.82	＊＊	2.30 ± 0.95	＊＊
VE 群	1.75 ± 0.44	2.05 ± 0.91	＊＊	2.20 ± 0.81	＊＊
VC/VE 群	1.77 ± 0.43	2.12 ± 0.85	＊＊	2.26 ± 0.89	＊＊
プラセボ群	1.64 ± 0.53	2.02 ± 0.80	＊＊	2.38 ± 0.97	＊＊

ベースラインと 12 週目，24 週目の比較；＊：$p<0.05$，＊＊：$p<0.01$(Wilcoxon の符号付順位検定)

(ANOVA) およびその後の多重比較検定によって解析を行った．群内の経時変化は，Bonferroni 修正の Wilcoxon の符号付順位検定で解析を行った．I CTP の改善・維持および悪化部位数の群間比較は，Pearson の χ^2 検定で検討した．ベースライン時の CAL または PD が 3 mm 未満の部位については，臨床的に健常またはそれに近いとみなされる部位として，特に詳細な解析を行った．試験期間中，歯肉縁下の処置を行った部位については解析対象からは除外した．

検定における有意水準は，χ^2検定の場合，片側 5%とし，そのほかの検定法では両側 5%とした．統計学的検討に際しては，統計計算ソフト SPSS® version 11.5.1 J for Windows (SPSS Japan，東京) を用いた．

結　果

1．症例全体の概況

採用された被験者 65 名のうち，4 名は被験者本人の理由により，24 週目の来院まで継続できなかったが，12 週目までの結果については解析に採用した．

各群の年齢，喫煙本数，喫煙歴，Packyear，現在歯数，食事からの VC 摂取量と VE 摂取量の試験期間中の平均値を表 3 に示した．これらの背景因子においては，各群間に有意な差は認められなかった．なお，試験期間中，タブレット摂取が直接の原因と思われる副作用は認められなかった．

2．臨床パラメータ

CAL, PD の被験者単位での平均の解析を行った結果，群内の経時的な変化および各診査時における群間比較において，有意差は認められなかった．したがって，以下の結果では，各部位単位の解析結果を示す．

1）CAL

表 4 a に各群の CAL 平均の経時的な変化を示した．24 週目では，ベースライン時と比較して，VC 群，VC/VE 群およびプラセボ群では有意な増加が($p<0.01$)，VE 群では有意な減少が認められた ($p<0.01$)．さらに健常またはそれに近い部位としてベースライン時の CAL が 3 mm 未満の部位に限って解析したところ (表 4 b)，ベースライン時と比較して 12, 24 週目ですべての群において有意な増加が認められた ($p<0.01$)．

各群ごとの CAL の 24 週目の変化量平均については表 5 に示した．全部位では VC 群，VE 群および VC/VE 群で，プラセボ群と比較して有意な悪化の抑制が認められた($p<0.05$)．特に VE 群は 24 週目で，ほかの群と比較し有意な CAL の改善を示した($p<0.01$)．ベースライン時の CAL が 3 mm 未満の部位においても，VC 群，VE 群および VC/VE 群はプラセボ群に対し有意な悪化抑制効果を示したが($p<0.01$)，このとき，VC 群，VE 群および VC/VE 群の群間に有意な差は認められなかった．

表 5　CAL 変化量（24 週目）の比較

群	全部位 (n=9,195)	群間比較				3 mm 未満の部位 (n=3,059)	群間比較			
	平均　標準偏差	VC	VE	VC/VE	プラセボ	平均　標準偏差	VC	VE	VC/VE	プラセボ
VC 群	0.08 ± 1.12		＊＊	NS	＊	0.56 ± 0.89		NS	NS	＊＊
VE 群	−0.18 ± 1.08			＊＊	＊＊	0.46 ± 0.81			NS	＊＊
VC/VE 群	0.08 ± 1.10				＊＊	0.49 ± 0.88				＊＊
プラセボ群	0.19 ± 1.21					0.74 ± 1.03				

変化量の群間の比較；＊：$p<0.05$，＊＊：$p<0.01$（ANOVA の後，Dunnett の検定）

表 6　VC および VE が PD およびその変化量に及ぼす影響

a）全部位

群	ベースライン(n=9,804)	12 W (n=9,804)		24 W (n=9,214)	
	平均　標準偏差	平均　標準偏差		平均　標準偏差	
VC 群	2.62 ± 1.12	2.62 ± 1.00		2.63 ± 1.06	
VE 群	2.76 ± 1.30	2.55 ± 1.24	＊＊	2.51 ± 1.18	＊＊
VC/VE 群	2.63 ± 1.24	2.60 ± 1.22		2.63 ± 1.26	
プラセボ群	2.63 ± 1.28	2.57 ± 1.17	＊＊	2.69 ± 1.21	＊＊

b）ベースライン時が 3 mm 未満の部位

群	ベースライン(n=5,092)	12 W (n=5,092)		24 W (n=4,890)	
	平均　標準偏差	平均　標準偏差		平均　標準偏差	
VC 群	1.70 ± 0.46	2.01 ± 0.66	＊＊	2.07 ± 0.78	＊＊
VE 群	1.73 ± 0.45	1.79 ± 0.66	＊＊	1.84 ± 0.63	＊＊
VC/VE 群	1.73 ± 0.45	1.91 ± 0.74	＊＊	1.94 ± 0.77	＊＊
プラセボ群	1.71 ± 0.47	1.86 ± 0.64	＊＊	2.07 ± 0.77	＊＊

ベースラインと 12 週目，24 週目の比較；＊＊：$p<0.01$（Wilcoxon の符号付順位検定）

また，図表には示さなかったが，ベースライン時と比較して 24 週時点で CAL の改善・維持が認められた部位の割合は，ベースラインの部位数を 100%としたとき，VC 群，VE 群，VC/VE 群でそれぞれ 53.5, 59.1, 58.3%となり，プラセボ群の 45.8%と比較して有意に高かった（$p<0.01$）．

2）PD

表 6 に各群の PD 平均の経時的な変化を示した．健常またはそれに近い部位としてベースライン時の PD が 3 mm 未満の部位に限って解析したところ（表 6 b），ベースライン時と比較して 12, 24 週目ですべての群において有意な増加が認められた（$p<0.01$）．

また，PD の各群ごとの変化量平均については，表 7 に示した．全部位では VC 群，VE 群および VC/VE 群のいずれの群においても 24 週目で，プラセボ群と比較して有意な悪化の抑制が認められた（$p<0.05$）．特に VE 群は 24 週目で，ほかの群と比較し有意な PD の改善作用を示した（$p<0.01$）．ベースライン時の PD が 3 mm 未満の部位では，24 週目で VE 群，VC/VE 群は，プラセボ群に対し悪化の抑制を示した（$p<0.01$）．また，図表には示さなかったが，ベースライン時と比較して 24 週時点で PD の改善・維持が認められた部位の割合は，ベースラインの部位数を 100%としたとき，VE 群，VC/VE 群でそれぞれ 80.6, 74.5%となり，プラセボ群の 63.8%と比較し有意に高かった（$p<0.01$）．

3）PI

被験者単位の平均の解析を行った結果，群内での経時的な有意な変化は認められなかった．しかし，各診査時における群間比較を行ったところ，12 週目において，プラセボ群と VC 群の間に有意差が認められた（p<

表 7　PD 変化量（24 週目）の比較

群	全部位 (n=9,214)	群間比較			3 mm 未満の部位 (n=4,890)	群間比較			
	平均　標準偏差	VC	VE	VC/VE	プラセボ	平均　標準偏差	VC	VE	VC/VE	プラセボ
VC 群	0.04 ± 0.89		**	NS	*	0.39 ± 0.74		**	**	NS
VE 群	−0.22 ± 0.82			**	**	0.12 ± 0.56			**	**
VC/VE 群	0.00 ± 0.85				**	0.21 ± 0.71				**
プラセボ群	0.12 ± 0.96					0.37 ± 0.78				

変化量の群間の比較；*：$p<0.05$，**：$p<0.01$（ANOVA の後，Dunnett の検定）

表 8　PI の比較

群	ベースライン (n=1,552)	12 W (n=1,552)		24 W (n=1,456)	⊿12 W (n=1,552)		⊿24 W (n=1,456)	
	平均　標準偏差	平均　標準偏差		平均　標準偏差	平均　標準偏差		平均　標準偏差	
VC 群	0.72 ± 0.69	0.83 ± 0.72	**	0.76 ± 0.69	0.12 ± 0.71	††	0.07 ± 0.76	#
VE 群	0.76 ± 0.74	0.70 ± 0.71		0.71 ± 0.68	−0.07 ± 0.65		−0.03 ± 0.60	
VC/VE 群	0.85 ± 0.75	0.79 ± 0.73		0.88 ± 0.75	−0.06 ± 0.68		0.03 ± 0.75	
プラセボ群	0.88 ± 0.67	0.74 ± 0.74	**	0.79 ± 0.74	−0.14 ± 0.56		−0.07 ± 0.64	

ベースラインと 12 週目の比較；**：$p<0.01$（Wilcoxon の符号付順位検定）
変化量の VC 群とほかの群との間の比較；††：$p<0.01$（ANOVA の後，Dunnett の検定）
変化量の VC 群とプラセボ群との間の比較；#：$p<0.05$（ANOVA の後，Dunnett の検定）

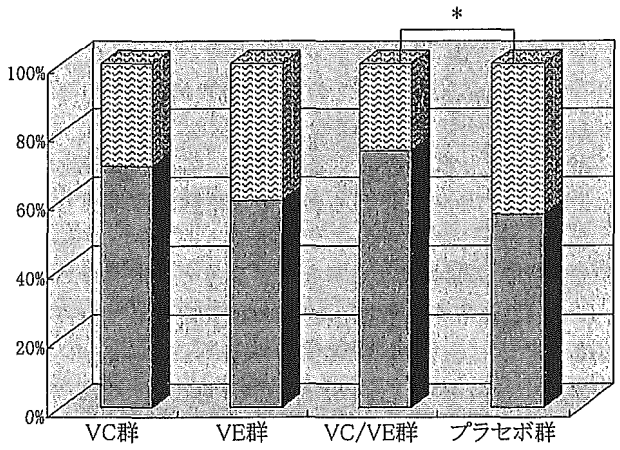
0.05）．

PI の全測定部位の平均について表 8 に示した．プラセボ群は，12 週目において有意に減少したが（$p<0.01$），24 週目では増加していた．VE 群，VC/VE 群においても，有意差はないものの同様の傾向が認められた．一方，VC 群はほかの群とは異なり，12 週目で有意に増加していた（$p<0.01$）．また，PI の変化量では，12 週目，24 週目で VC 群がほかの群と比較して有意に高いことがわかった．

3．GCF 中の I CTP および血中生化学的マーカー

図 1 にベースライン時と比較して 24 週時点で GCF 中の I CTP の改善・維持が認められた部位数と悪化した部位数の比較を示した．VC/VE 群の改善・維持部位の割合は 74.5%と，プラセボ群の 56.0%に比較して有意に高かった（$p<0.05$）．

血中の VC，VE レベルを図 2，3 に示した．12 および 24 週目の血中の VC 濃度は，VC 群，VC/VE 群で（$p<0.01$），また VE 濃度は試験期間中 VE 群，VC/VE 群で有意に増加していた（$p<0.05$）．

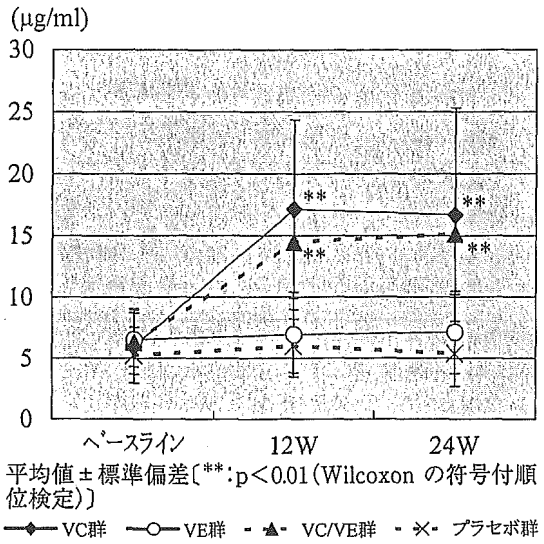


*: $p<0.05$（Pearson の χ^2 検定）　▨改善・維持部位　▩悪化部位

図 1　GCF 中の I CTP の改善・維持および悪化部位数の割合

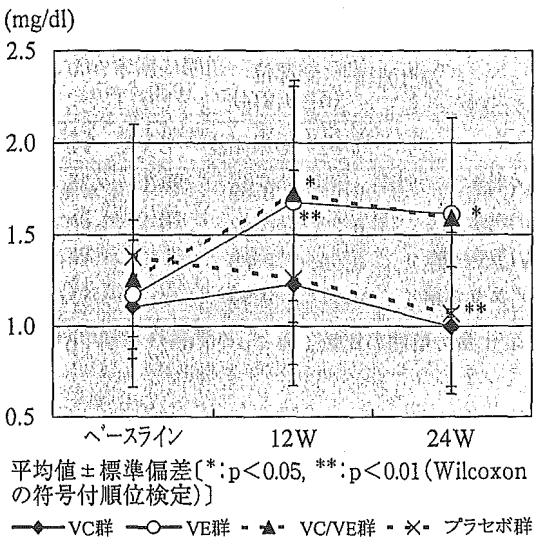
考　察

近年，歯周病と全身の関係について，縦断的・横断的な疫学研究の知見が蓄積されてきた結果，喫煙が歯周病の危険因子の 1 つである可能性はかなり高いと考えられている[4]．歯科医による患者への禁煙指導は，歯周病の発症や進行のリスクを排除するうえで最も重要と思われる．しかし，一般的な外来診療の場での禁煙指導における成功率は 16〜20%に留まっており[18,19]，また禁煙を長



平均値±標準偏差〔**:p<0.01(Wilcoxon の符号付順位検定)〕

図 2　血中 VC 量の変化



平均値±標準偏差〔*:p<0.05, **:p<0.01(Wilcoxon の符号付順位検定)〕

図 3　血中 VE 量の変化

期的に維持できる者は決して多くない．禁煙指導は第一に実施されなければならない予防的介入であることはいうまでもないが，本臨床試験は，禁煙に無関心であったり，禁煙にまでいたらなかった者の歯周病に対する喫煙リスクの減少に寄与する一方法として，VC，VE のサプリメンテーションの可能性を探ることを目的とした．

今回の試験結果から，抗酸化ビタミンである VC や VE は，単独においても，その併用においても，歯周病の進行に対し，臨床的な抑制効果をもつことが示唆された．VE 群は，試験製剤群全体のなかで特に有効性が高かったが，一方で VC 群は顕著な効果を示さなかった．また，VC と VE はともに摂取することで，相乗的抗酸化作用が生じることから，これらの併用は単独摂取よりも効果的であると予測されたが，今回の CAL と PD における試験製剤投与の結果は，予測と異なっていた．その理由として，被験者の口腔衛生管理や背景因子などの違いが CAL や PD に影響した可能性が考えられる．たとえば，12 週目において，プラセボ群やほかの試験製剤群の PI がいずれも減少傾向であるのに対して，VC 群の PI は増加していることが分かる．このことから，他群は試験に参加することで口腔衛生習慣が動機づけされたにもかかわらず，VC 群はむしろ悪化したと考えられる．また，その後 24 週目にかけて VC 群の PI は改善し，一方そのほかの群では悪化したが，試験終了時の VE 群とプラセボ群の 2 群の PI は，ベースライン時と比較して依然低かった．以上のことから，偶然の結果とは思われるが，試験期間中の口腔衛生の状態には群間で差があったものと考えられる．

試験期間中の CAL の測定部位単位の変化量は，被験者単位の PI の変化量と有意な相関性（Pearson の相関係数，$p<0.01$）が認められたが，PI の変化量が±0.15 以下の被験者に限って解析した場合には相関性は認められなかった．これらの被験者について，再度全部位の CAL の変化量の群間差を解析してみたところ，VC 群，VE 群および VC/VE 群とプラセボ群の間には有意な差（$p<0.01$）が認められたが，試験製剤群の 3 群の間には有意差は認められなかった．このことから，PI の変化の影響を除いた時の CAL への有効性において，VC，VE およびその併用摂取は，同等であったと考えられる．また，有意差は認められなかったものの，VE 群は平均年齢がほかの群と比べ低く，結果的に喫煙習慣の総合的な指標となる Packyear が他群と比較して小さい傾向が認められた．以上を考え合わせると，プラーク沈着の試験期間中の変化は，試験期間中の歯周病の状態に影響を及ぼし，VC 群や VC/VE 群において製剤の働きを相殺した可能性が，また VE 群においては他群よりも年齢や喫煙のリスクが低かったことにより，VE の作用を助長した可能性が考えられた．

本研究では特に健常またはそれに近い部位への試験製剤の影響を調べる目的で，ベースライン時の CAL または PD が 3 mm 未満の部位に限った解析を行った．プラセボ群において示されるように，試験期間中これらの部位の値は有意に増加しており，悪化傾向が認められた．本試験では，試験製剤の摂取により，これらの部位の CAL や PD の増加が抑制されたことから，VC，VE およびその併用摂取は喫煙者の歯周病の発症や初期での進行に対しても，予防的な作用がある可能性が示唆された．

今回の試験では，歯周病の生化学的な指標として，

GCF中のICTPについても測定を行った．血中ICTPは，骨代謝と関連性が高いため，骨髄腫や骨転移，閉経後女性の骨粗鬆症の生化学的なマーカーとして用いられる．ICTPのGCFにおけるレベルは，歯周炎患者において有意に高く，PDや歯槽骨吸収などの臨床パラメータとの関連性も認められたという報告がある[20]．本研究の被験者のベースライン時におけるICTP量は237.7 pg/siteと，ほかの文献で報告されている歯周病罹患部位の124～350 pg/siteの範囲内にあった[21,22]．GCF中のICTPは，骨吸収を予測する指標として[17]，また初期治療や化学療法などの効果を予測する指標として有用性が高いことを示す報告[21,22]があり，今回の試験でVC/VE群において，ICTP改善・維持部位の割合がプラセボよりも有意に高かったことは，VCとVEの併用摂取が，将来的な改善に寄与することを示している．

喫煙者の血中VC量は，非喫煙者と比較して低いことが知られている[23]．今回の被験者全体のベースライン時における血中VC量は，平均的なVC量を通常の食事から摂取していたにもかかわらず（91.1 mg±34.5 mg/日），6.0 μg/mlと基準値の5.5～16.8 μg/mlの下限に近く，VCの消耗が促進されていることが示された．Seriら[24]の報告では，歯周病学的に健常な喫煙者から得たGCF中のVC濃度は，非喫煙者の濃度よりも有意に低く，またVEのレベルにも低下が認められた．またTüterら[25]は，慢性歯周炎患者の歯周組織中のチオバルビツール酸反応物質（Thiobarbituric acid reactive substances：TBARS）レベルが，歯周病の状態と強い関連性のあることを報告している．病原菌と宿主の間の反応より発生するROSは，DNA損傷やタンパク質の変性，重要な酵素類の酸化，炎症性サイトカイン産生促進，脂質の酸化など多くの作用機序を介して，組織傷害の原因となっている[26]．ROSの最も一般的な評価法であるTBARSの組織中の増加は，ROSが歯周組織損傷のメカニズムの1つとして影響を及ぼしていることを示唆している．喫煙者の場合，ROSがたばこ煙から体内に取り込まれたり，産生が促進されることで，血中のTBARSレベルは非喫煙者よりも高くなっており[27]，その消去に必要とされるVCなどの抗酸化物の消耗も大きいと考えられる．このことから，試験製剤によるVCやVEの摂取が，血流を介して歯周組織中のVC，VE濃度を改善し，歯周組織中のROSを消去する抗酸化能を高めた可能性が考えられる．さらに，その結果，歯周病進行の抑制に対し，CALやPDにおいて臨床的な効果をもたらしたのかもしれない．本研究では喫煙者のみを被験者としたが，歯周病進行のメカニズムの1つとして，ROSなど酸化ストレスの影響があると考えると，喫煙習慣のない歯周病罹患者に対しても，抗酸化ビタミンの摂取は歯周病進行に対する予防的な作用を及ぼすことも考えられる．

抗酸化物，特にVCやVE摂取の歯周病への有効性を示唆した臨床試験結果の報告はあまり多くない．VEについては報告がなく，VCについては，VC欠乏状態によって歯肉の炎症が惹起し，VC補充以降改善作用が認められた臨床試験結果が報告されているが[12,13]，いずれも健常な歯周組織を有する非喫煙者を対象としていた．本試験では，被験者単位では有意な作用を示すことができなかったものの，緩徐なサプリメントの作用を限られた被験者数で検証するために，測定部位単位で解析を行った場合では，抗酸化ビタミン摂取の有効性を示唆することできた．今回の結果を踏まえ，今後は被験者数や試験期間を拡大した研究へ展開していく必要があるだろう．

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Effect of Vitamin C and Vitamin E Supplements on Periodontal Disease in Smokers

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Abstract : Smoking habit has been reported as a risk factor for periodontal disease and low vitamin C intake has been associated with periodontal status. In this randomized controlled trial, we investigated the efficacy of dietary supplementation with antioxidative vitamins, vitamins C and E, on suppression of the progression of periodontal disease in smokers.

A total of 65 patients who were current smokers were randomly assigned to one of the following groups : 1) VC (1,000 mg/day) ; 2) VE (135 mg/day) ; 3) combination of VC and VE ; 4) placebo. Plaque index (PI), clinical attachment level (CAL), pocket depth (PD) and cross-linked carboxyterminal telopeptide of type I collagen (I CTP) from gingival crevicular fluid (GCF) as a biomarker of periodontal disease, were measured at baseline, 12 and 24 weeks following the start of supplementation.

CAL and PD in VC, VE and the combination groups showed significant improvements during this trial comparing with the placebo group ($p<0.05$). Furthermore, in the combination group, the rate of GCF samples in which I CTP was decreased or maintained was significantly greater than that in the placebo group at 24 weeks ($p<0.05$). On the other hand, VC levels in blood samples from VC and the combination groups ($p<0.01$) and VE levels from VE and the combination groups significantly rose from each level at baseline ($p<0.05$).

These results suggest that supplements of VC and VE may maintain the periodontal health status in smokers by controlling oxidative status and other mechanisms.

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Key words : **Risk factor for periodontal disease, Smoking habit, Vitamin C, Vitamin E, Randomized controlled trial**

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Detection of *Campylobacter rectus* in periodontitis sites by monoclonal antibodies

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Campylobacter rectus, a gram-negative, microaerophilic, and motile bacterium, has been proposed to play a pathogenic role in human periodontitis. Surface components, such as the flagellum, surface layer (S-layer), and cytotoxin, have been reported as possible virulence factors of the microorganism. In the present study, monoclonal antibodies against surface components of this bacterium were produced to detect and investigate the pathogenic potential of *C. rectus* in periodontitis. Two monoclonal antibodies, designated CRT-1 and CRT-2, recognized a peculiar 150 kDa S-layer protein by immunoblot analysis. The CRT-2 antibody reacted to all *C. rectus* strains tested, except for the S-layer negative strain of the species [*C. rectus* ATCC 33238 S-layer (–) strain]. The CRT-3 antibody reacted to a 60-kDa protein in *C. rectus* and also cross-reacted with *Campylobacter showae* ATCC 51164 and CCUG 11641 strains. Using the dot-blot method, we were able to detect *C. rectus* using the CRT-2 antibody when as few as 10^3 organisms were present in a subgingival dental plaque sample. Detection of *C. rectus* in plaque samples correlated significantly with clinical findings such as probing depth ($P < 0.001$), bleeding on probing ($P < 0.001$), and gingival index ($P < 0.001$). These findings indicate that infection by *C. rectus* may be an important indicator of periodontal disease status.

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Key words: *Campylobacter rectus*; monoclonal antibody; dot-blot analysis; periodontal sites

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Recent microbial studies and clinical examinations have revealed that specific gram-negative bacteria are essential pathogenic factors in various types of human periodontal disease. In the diagnosis of periodontal disease, microbial examinations can identify patients and periodontal sites that are undergoing active tissue destruction and can aid in understanding of fluctuations of periodontopathogens after treatment (1–3). *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Eikenella corrodens*, *Bacteroides forsythus*, *Campylobacter rectus*, and *Treponema denticola* are known as major periodontal bacteria. *Campylobacter rectus* has often been detected in large numbers in deeper subgingival pockets (4–6) and has been implicated in adult periodontitis and rapidly advancing periodontitis. It has been reported that, in adult periodontitis, these organisms were detected more frequently than *P. gingivalis* or *A. actinomycetemcomitans* by using PCR methods (7).

Campylobacter rectus was previously called *Wolinella recta*; it was renamed by Vandamme *et al.* (8). It has a flagellum and motility, and it is a gram-negative, microaerophilic, round-ended, straight, non-glycolytic bacterium (9, 10). This organism is supposed to reach the depths of periodontal pockets using the motility of its flagellum, which appears to be a major pathogenic factor, but which has not yet been purified, unlike the fimbria of *P. gingivalis*. Electron microscopic studies (11–14) have shown that *C. rectus* possesses a characteristic cell surface layer composed of hexagonally arrayed subunits. *Campylobacter curvus*, *C. showae*, *Wolinella succinogenes*, and other *C. rectus* related organisms do not have this S-layer; it is a protein peculiar to *C. rectus*, and it has been thought to be a major pathogenic factor (15, 16). Kesavalu *et al.* (17) demonstrated that an S-layer positive strain has a great ability to form abscesses than the negative strain in a murine model. Gillespie *et al.* (18, 19) reported that *C. rectus* secreted the cytotoxin of leukocyte resistance factor. Various pathogenic factors have

been nominated, but all of the details of the pathogenicity have not been determined. A few reports for clinical status and *C. rectus* were submitted. *Campylobacter rectus* has been reported to be detected from deep periodontal pockets (20–24), but pathogenic factors scientifically supporting these reports have not clarified. To detect periodontal pathogens for the purposes of diagnosis of periodontal disease and clinical assessment, there are many methods, including culture (25), fluoroimmunoassay (26), and detection using a DNA probe (27) and PCR (7); the latter are now the first choices for clinically estimating microorganisms. PCR is superior for specific bacteria; its detection limits are about 10^2 cells, and it is highly sensitive, but it requires special equipment. On the other hand, it is time consuming and very difficult to identify *C. rectus* by culture, so this method is not useful. Therefore, the aims of this study were first to prepare highly specific monoclonal antibodies against surface components with a high antigenic epitope and second to establish a dot-blot analysis method using these specific monoclonal antibodies against *C. rectus* surface components to enable *C. rectus* detection rapidly, simply, and easily with high specificity; third to examine the relationship between existence of and depth of periodontal pockets or bleeding on probing (BOP).

Materials and methods

Bacterial cultivation

Campylobacter rectus was grown in broth medium containing 1.0% trypticase peptone (Becton Dickinson and Company, Franklin Lakes, NJ, USA), 0.3% phytone peptone (Becton Dickinson), 0.2% yeast extract (Becton Dickinson), 37.4 mM ammonium chloride (Wako Pure Chemical Industries, Osaka, Japan), 44.1 mM sodium formate (Wako), 25.0 mM sodium fumarate (Wako), and 26.6 mM L-asparagine monohydrate (Nacalai Tesque, Kyoto, Japan) (pH 7.8) in an anaerobic atmosphere for 3 d at 37°C. Cells were precipitated by centrifugal separation (10,000 *g*, 20 min), washed three times in phosphate-buffered saline (PBS), and then used as whole cell antigens.

Preparation of monoclonal antibodies

Monoclonal antibodies were prepared by the method of Köhler and Milstein (28). *Campylobacter rectus* ATCC 33238 whole cells were used as the immunogen; five-week-old female BALB/c mice were immunized once a week for four weeks. Mice first received a few subcutaneous injections of 80 μg of a whole-cell suspension in PBS with an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA); second and third injections were carried out intraperitoneally. The spleen and the thymus were extracted from those mice whose antibody titers rose 3 d after the final immunization. Mouse myeloma (SP2) and spleen cells were fused by 1 g/ml polyethylene glycol 4000 (Sigma Chemical Company, St. Louis, MO, USA), and the fused hybridomas were incubated with the thymus. Hybridomas were selectively cultured in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) broth medium including 10% fetal calf serum (FCS, Bio Whittaker, Walkersville, MD, USA), 0.1% $NaHCO_3$ (Wako), 10 mM HEPES (Sigma), 0.01% streptomycin sulfate (Meiji, Tokyo, Japan), and 0.03% glutamine (Nissui) added to 1.0×10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine, and 4.0×10^{-7} M aminopterine. Hybridomas that produced antibodies against *C. rectus* were screened by enzyme-linked immunosorbent assay (ELISA) and cloned by the limiting dilution method. The selected clones were preserved by freezing in liquid nitrogen. These hybridoma culture supernatants were used as monoclonal antibodies. Reactivity of these antibodies was evaluated by the ELISA method using 96-well microtiter plates (Corning, Corning, NY, USA). All of this study followed the animal experimental principles of Tokyo Dental College.

Determination of monoclonal antibodies isotypes

Monoclonal antibody isotypes were determined by using a monoclonal subisotyping kit (American Qualex Company, San Clemente, CA, USA).

Verification of specificity of the monoclonal antibody

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the methods of Laemmli (29) in 10–20% gradient resolving gel (Daiichi-Kagaku, Tokyo, Japan) Before PAGE, samples and marker proteins were treated with 2% SDS and 5% 2-mercaptoethanol at 100°C for 3 min. After PAGE the electrophoresed proteins were transferred by the method of Towbin *et al.* (30) with a Transblot cell (Bio-Rad, Hercules, CA, USA). The blotted membranes were immunostained with the monoclonal antibodies. Antibody bound to protein immobilized on the membranes was detected with peroxidase-conjugated goat antimouse immunoglobulins (Bio-Rad). We examined seven *C. rectus* strains (two standard strains: ATCC 33238 S-layer (+), and S-layer (–) strain, which was kindly provided from Dr S. Kokeguchi; and five clinical isolates: 306, 325, 640, 632, 796), *B. forsythus* ATCC 43037, *A. actinomycetemcomitans* ATCC 43718, *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 25586, *Prevotella nigrescens* ATCC 33563, *P. intermedia* ATCC 25611, *Helicobacter pylori* ATCC 43504, and *W. succinogenes* ATCC 29543. Verification of specificity among other *Campylobacter* species was performed using strains of *C. rectus*, *C. showae* ATCC 51164, CCUG 11640, *C. curvus* ATCC 35224, *C. concisus* ATCC 33237, and *C. sputorum* ATCC 35980 (This strain is supplied by Department of Oral Microbiology, Showa University School of Dentistry).

Immunoelectron microscopy

Whole cells of *C. rectus* ATCC 33238 were washed three times with PBS, resuspended in PBS, and fixed on a mesh on which collogion-membrane had been spread. The mesh was incubated with CRT-2 for 1 h at room temperature, washed with PBS three times, and reacted with Gold Conju-

gated antibody [EM Goat anti-Mouse IgG (British Biocell International, Cardiff, UK)] for 1 h at room temperature. It was washed three times, negatively stained with 1% (w/v) phosphotungstic acid, washed, and observed electron microscopically.

Dot-blot analysis

On a nitrocellulose membrane (Millipore Co. Ltd, Tokyo, Japan), 1 μl of each sample was dotted and dried. The membrane was blocked with PBS-skim milk for 45 min. The membrane was incubated in CRT-2 diluted 1 : 100 in PBS-skim milk for 45 min. It was washed twice briefly with distilled water and once with PBS-skim milk for 2 min, incubated for 45 min in 1000-fold-diluted peroxidase-conjugated goat antimouse immunoglobulins. It was washed again with distilled water, and finally incubated with PBS-0.05% Tween (Tween 20, Nacalai Tesque) for 2 min. Then it was washed five times with distilled water, and the development was performed until the antibody bearing dots were visible. Cross-reactivity of other species with *C. rectus* monoclonal antibodies and the sensitivity of the test was investigated by dot-blot analysis. It took 2.5 h for detection, so this method takes less than half the conventional method (about 6 h). To examine cross-reactivity with other species, an antigen–antibody reaction was performed onto a membrane on which five species (*P. gingivalis* ATCC 33277, *A. actinomycetemconitans* ATCC 43718, *B. forsythus* ATCC 43037, *H. pylori* ATCC 43504, *Escherichia coli* HB101) were dotted. Detection limits were examined by the dot-blot analysis in the same manner. Briefly, 1 μl of suspensions of *C. rectus* whose numbers of cells had been adjusted from 10^1 to 10^5 cells/μl was spotted on the membrane.

Confirmation of detection of *C. rectus* by PCR

Detection of *C. rectus* was confirmed by the PCR described by Ashimoto *et al.* (7). In this study, 5′-TTT CGG AGC GTA AAC TCC TTT TC-3′ and 5′-TTT CTG CAA GCA GAC ACT CTT-3′ were used as primers (7).

Clinical application of dot-blot analysis with monoclonal antibodies

Subjects — Individuals examined in this study were patients with adult periodontitis from the Department of Periodontics, Tokyo Dental College. Twenty-four patients (six males and 18 females; mean age, 50.7 years) were selected based upon radiographical evidence of bone loss and the presence of periodontal pockets (≥ 5 mm). These patients had not taken any systemic antibiotics during the previous 6 mo.

Clinical examinations — Clinical examinations included the pocket depth measured with the William's probe, gingival index (GI), pocket depth (PD), and bleeding on probing (BOP) (31). The positive criterion for BOP was gingival bleeding within 30 s after probing. Gingival inflammation was assessed using the GI index of Löe and Silness (32).

Sampling — Sample sites were isolated with cotton rolls. After removing the supragingival plaque with sterile cotton pellets, three paper points (Pierce Absorbent Points #40, Pierce Dental, Tokyo, Japan) were inserted into the bottom of each periodontal pocket for 30 s. After removal, they were placed into 100 μl of sterilized PBS and stored at −80°C until use. When used for detection, thawed samples were centrifuged. After impurities such as blood ingredients were removed, the samples were resuspended in 50 μl of PBS and then subjected to dot-blot analysis.

Statistical analysis

Specificity, sensitivity, and accuracy were analyzed by the method of Gladen (33). Briefly, the specificity was calculated as the number of dot-negative and PCR-negative (true negative) sites divided by the negative results in the PCR method. The sensitivity was estimated as the number of dot-positive and PCR-positive (true positive) sites divided by

Table 1. Reactivity of three monoclonal antibodies with *C. rectus* strains and other bacterial species

	Monoclonal antibody CRT-1	CRT-2	CRT-3
	Isotype IgGI	IgG2a	IgG2b
Campylobacter rectus ATCC 33238 S-layer (+)	+	+	+
Campylobacter rectus ATCC 33238 S-layer (–)	–	–	+
Campylobacter rectus 640	+	+	+
Campylobacter rectus 632	–	+	+
Campylobacter rectus 306	+	+	+
Campylobacter rectus 325	–	+	+
Campylobacter rectus 796	+	+	+
Campylobacter showae ATCC 51164	–	–	+
Campylobacter showae CCUG 11641	–	–	+
Campylobacter curvus ATCC 35224	–	–	–
Campylobacter concisus ATCC 33237	–	–	–
Campylobacter sputorum ATCC 35980	–	–	–
Bacteroides forsythus ATCC 43037	–	–	–
Actinobacillus actinomycetemcomitans ATCC 43718	–	–	–
Porphyromonas gingivalis ATCC 33277	–	–	–
Fusobacterium nucleatum ATCC 25586	–	–	–
Prevotella nigrescens ATCC 33563	–	–	–
Prevotella intermedia ATCC 25611	–	–	–
Treponema denticola ATCC 33520	–	–	–
Treponema denticola ATCC 35405	–	–	–
Helicobacter pylori ATCC 43504	–	–	–

+; positive reaction; –: negative reaction.

the positive results in the PCR method. The agreement was estimated by the number of true positive plus true negative sites divided by the total number of results. Differences between microbiota detection and clinical characteristics in the initial examination were estimated by chi-squared test.

Results

The specificity of monoclonal antibodies

Reactivity of the standard strain and the clinical strains — Three monoclonal antibodies were obtained and named CRT-1, CRT-2, and CRT-3. CRT-1, whose isotype was IgG1, reacted with the *C. rectus* S (+) strains and the clinical strains (640, 306, 796) and recognized a peculiar 150 kDa protein by immunoblot analysis, but did not react with the S-layer (–) strains, 632 and 325 (Table 1). CRT-2, whose isotype was IgG2a, reacted with the *C. rectus* S (+) strain and all clinical isolates (Fig. 1a, b) and recognized a peculiar 150 kDa protein except in S (–) strains. CRT-3, whose isotype was IgG2b (Table 1), reacted with all the *C. rectus* strains tested and recognized a specific 60 kDa protein (data not shown).

Reactivity of monoclonal antibodies with other Campylobacter species and other bacterial species — None of the monoclonal antibodies cross-reacted with other bacteria (Table 1). CRT-1 and CRT-2 did not react to Campylobacter species other than *C. rectus*, but CRT-3 cross-reacted with *C. showae* (Table 1).

Immunoelectron microscopy

The localization on the bacterium of the antigen reacting with CRT-2 was confirmed by immunoelectron microscopy (Fig. 2). Gold particles on the antibody combined with the S-layer near the surface of the cell reacted with CRT-2. On the other hand, fewer gold particles were observed on the surface of control serum reacted *C. rectus* cell.

Detection of *Campylobacter rectus* with CRT-2 by dot-blot analysis

Cross-reactivity of other species with CRT-2 by dot-blot analysis — CRT-2 reacted only to *C. rectus*; it did not react to *P. gingivalis* ATCC 33277, *A. actinomycetemcomitans* ATCC 43718, *B. forsythus* ATCC 43037, *H. pylori* ATCC43504, or *E. coli* HB101 (Fig. 3).

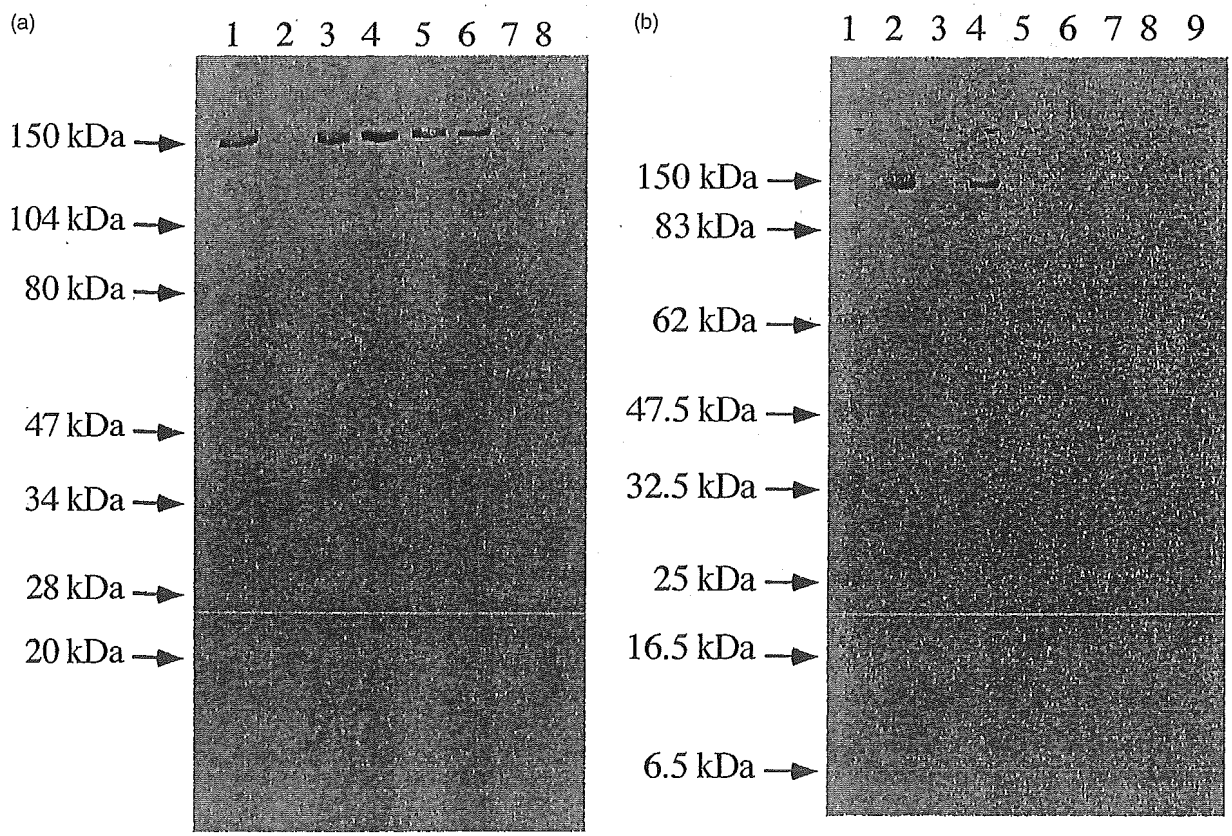

Fig. 1. (a) Reactivity of CRT-2 monoclonal antibody with a *C. rectus* clinical isolate. Lane 1: *C. rectus* ATCC 33238 S-layer (+); lane 2: *C. rectus* ATCC 33238 S-layer (–); lane 3: *C. rectus* 674; lane 4: *C. rectus* 632; lane 5: *C. rectus* 325; lane 6: *C. rectus* 306; lane 7: *W. succinogenes* ATCC 29543; lane 8: *C. rectus* 796. (b) Reactivity of CRT-2 monoclonal antibody with *Campylobacter* genus. Lane 1: molecular weight marker; lane 2: *C. rectus* ATCC 33238 S-layer (+); lane 3: *C. rectus* ATCC 33238 S-layer (–); lane 4: *C. rectus* CCUG 19168; lane 5: *C. showae* ATCC 51146; lane 6: *C. showae* CCUG11641; lane 7: *C. curvus* ATCC 35224; lane 8: *C. concisus* ATCC 33237; lane 9: *C. sputorum* ATCC 35980.

Detection of C. rectus *in dot-blot analysis* — CRT-2 positively reacted to dots containing 10^5 to 10^3 cells/μl and reacted negatively to dots containing 10 to 10^2 cells (Fig. 4). Therefore, from the dot-blot analysis, the detection limit of *C. rectus* was established at 10^3 cells. Dot-blot analysis of *C. rectus* from subgingival plaque of periodontitis affected subjects were shown in Fig. 5. The plaque samples from periodontal pockets were detected by this blot system. The result of detection was agreed with PCR detection (data not shown). Sample numbers 1 and 2 had showed positive reactions; number 3 had a negative reaction.

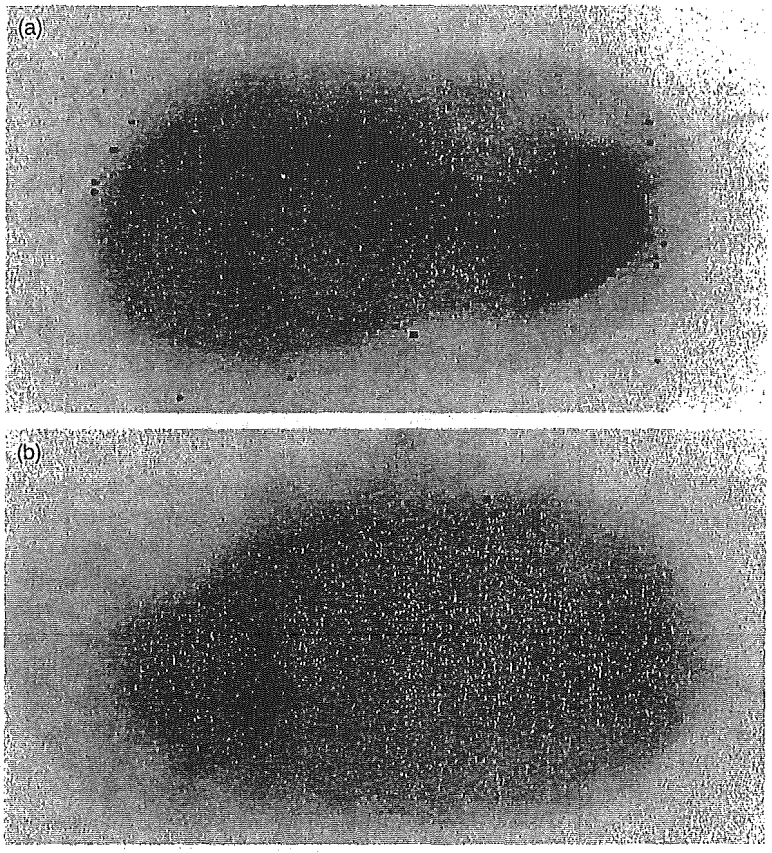

Fig. 2. (a) Immunoelectron microscopy of *C. rectus* ATCC 33238 strain stained with CRT-2 antibodies. Gold particles can be seen on the surface of the cells. (b) Negative control stained with control serum.

Comparison of detection rate of *C. rectus* between PCR and dot-blot analysis using CRT-2

Agreement between PCR and dot-blot analysis was 68.2%, specificity was 56.9%, sensitivity was 80.3% (Table 2). The detection rate of *C. rectus* detected by dot-blot analysis but not by PCR was 22.3%; that detected by PCR but not by dot-blotting was 9.5%.

Clinical examination at the time of sampling of plaques

The means and the standard deviations of the clinical conditions of sample sites are summarized in Table 3. The differences in PD were classified into three categories. The mean and standard deviation of PD was 1.93 ± 0.2 in the PD ≤ 2 group, 4.09 ± 0.8 in the 3 ≤ PD ≤ 5 group, and 6.98 ± 1.2 in the PD ≥ 6 group ($n = 211$). The BOP positive ratio, which was calculated from the number of BOP positive vs. the total number of patients, was 27.8% in the PD ≤ 2 group, 59.3% in the 3 ≤ PD ≤ 5 group, and 96.5% in the PD ≥ 6 group (Table 3).

Relationship between PD and the detection rate of C. rectus *by dot-blot analysis* — Ten out of 46 sites in the PD ≤ 2 group were *C. rectus* positive; the detection rate was 21.7%. In the 3 ≤ PD ≤ 5 group, 64 out of 108 sites were positive (59.3%), and in the PD ≥ 6 group, 55 out of 57 sites were

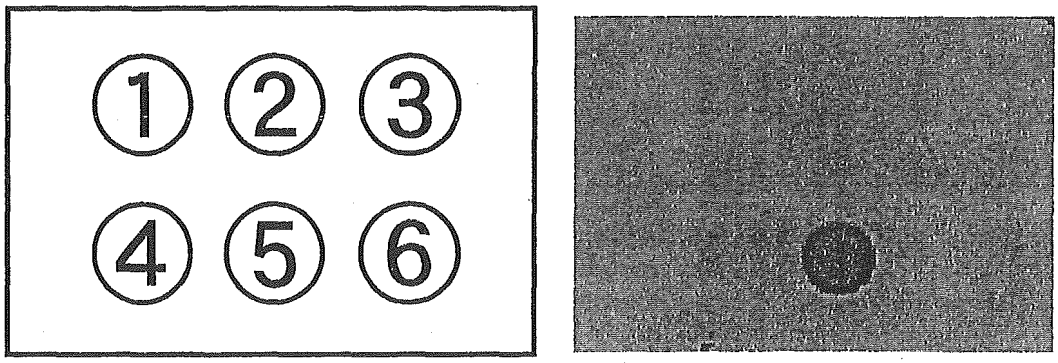

Fig. 3. Cross-reactivity of other species with CRT-2 by dot-blot analysis. 1: *P. gingivalis* ATCC 33277; 2: *A. actinomycetemcomitans* ATCC 43718; 3: *B. forsythus* ATCC 43037; 4: *H. pylori* ATCC 43504; 5: *C. rectus* ATCC 33238; 6: *E. coli* HB101.

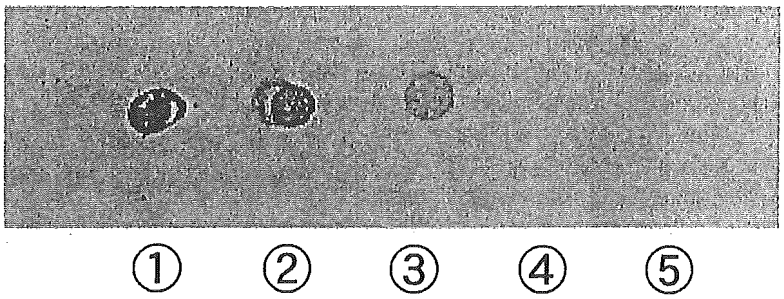

Fig. 4. Detection limits in dot-blot analysis. One microliter of suspensions of *C. rectus* in which the numbers of cells were adjusted from 10^1 to 10^5 cells/μl was spotted on the membrane. The dot-blot analysis detection limits for *C. rectus* were established at 10^3 cells. Cell numbers of *C. rectus*: 1: 10^5; 2: 10^4; 3: 10^3; 4: 10^2; 5: 10^1 cells/μl.

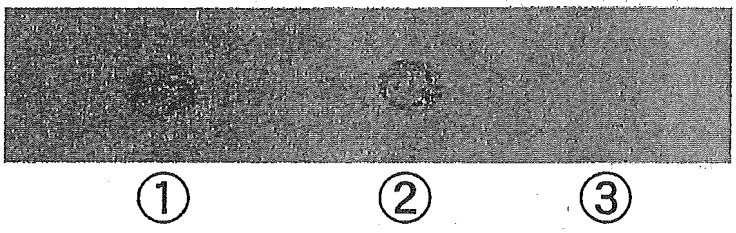

Fig. 5. Dot-blot analysis of *C. rectus* from a subgingival plaque sample from a patient with periodontitis. Samples 1 and 2: *C. rectus* positive; sample 3: *C. rectus* negative.

Table 2. Comparison between PCR and dot-blot analysis in detection of *C. rectus*

		PCR				
		Positive	Negative	Specificity	Sensitivity	Agreement
Dot-blot	Positive	82	47			
	Negative	20	62	56.9%	80.3%	68.2%

Detection of *C. rectus* by PCR was performed by the method described by Ashimoto *et al.* (7). PCR primers were 5′-TTT CGG AGC GTA AAC TCC TTT TC-3′ and 5′-TTT CTG CAA GCA GAC ACT CTT-3′.
$n = 211$.

positive (96.5%) (Fig. 6). The differences in detection rate with PD among each group were statistically significant ($P < 0.001$).

Relationship between BOP and the detection rate — The detection rate of *C. rectus* in BOP positive group was 76.5%; in the BOP negative group, it was 24.2% (Fig. 7). This difference was statistically significant ($P < 0.001$).

Relationship between GI and the detection rate — In the GI: 0 and GI: 1 groups, the detection rates were 21.1% and 26.4%, respectively; there was no significant difference (Fig. 8). However, between GI: 0 and GI: 2 (77.9%) and between GI: 0 and GI: 3 (88.5%), the differences were statistically significant ($P < 0.001$).

Discussion

Campylobacter rectus has been reported to be detected from deep periodontal pockets (20–24). Because it is as difficult to cultivate this organism as other periodontopathogens such as *B. forsythus* or *T. denticola*, its biochemical properties and pathogenic factors have not been well analyzed. It has been demonstrated that its pathogenic factors include an S-layer, which is a surface component, and a cytotoxin (18, 19), which is known to be a leukocytotoxic resistant factor and to weigh 104 kDa, but no molecular

Table 3. Initial clinical status of sampling sites in patients

PD	Mean ± SD	Ratio of BOP + (%)
≤ 2 mm	1.93 ± 0.2 ($n = 74$)	27.8%
3~5 mm	4.09 ± 0.8 ($n = 105$)	59.3%
≥ 6 mm	6.98 ± 1.2 ($n = 32$)	96.5%

$n = 211$.

biological studies have yet been performed. In the present study, we isolated monoclonal antibodies specific to *C. rectus* and developed a simple and convenient method which can detect the organism in clinical samples. In the current study, three monoclonal antibodies, which were named CRT-1, CRT-2 and CRT–3, were obtained. CRT-1 recognized a peculiar 150 kDa protein and reacted with *C. rectus* ATCC 33238, an S-layer (+) strain, but not with an S-layer (–) strain; therefore, CRT-1 is probably a monoclonal antibody against one of epitopes of the S-layer. The fact that some *C. rectus* strains tested by immunoblot analysis reacted to CRT-1 but others did not, showed that the S-layer is heterogeneous in clinical strains (34). CRT-2 reacted to the S-layer (+) strain and all our clinical isolates; it recognized a peculiar 150 kDa protein but did not cross-react with other species. Consequently, CRT-2 was considered to be a *C. rectus* specific monoclonal antibody. In the oral cavity, S-layer (+) strains predominate over the S-layer (–) mutant strain in the laboratory. Thus, *C. rectus* in plaque samples was detected specifically by CRT-2. CRT-3 reacted with all *C. rectus* strains, including S (–) ones, and recognized a peculiar 60 kDa protein. It also cross-reacted with both *C. showae* ATCC 51164 and CCUG 11641. *Campylobacter showae*, which also belongs to the Campylobacter genus, was detected in subgingival plaque by Etoh *et al.* (16). Strains of *C. showae* have from two to five flagella, but do not possess an S-layer; their biochemical properties may be different from those of *C. rectus* strains. The common 60 kDa antigen recognized by CRT-3 between *C. rectus* and *C. showae* may be elucidated by further analysis.

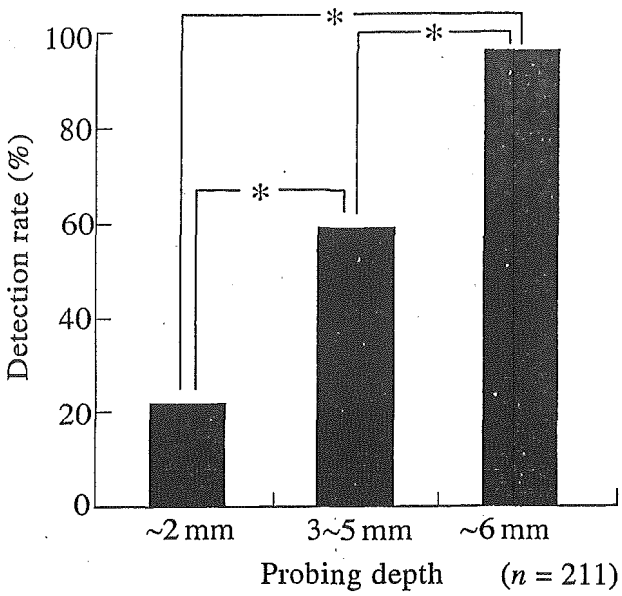

Fig. 6. Relationship between PD and the detection rate of *C. rectus*. The detection rate was significantly different at different probing depths (*$P < 0.001$ by chi-squared test). The greater the depth, the higher the infection rate.

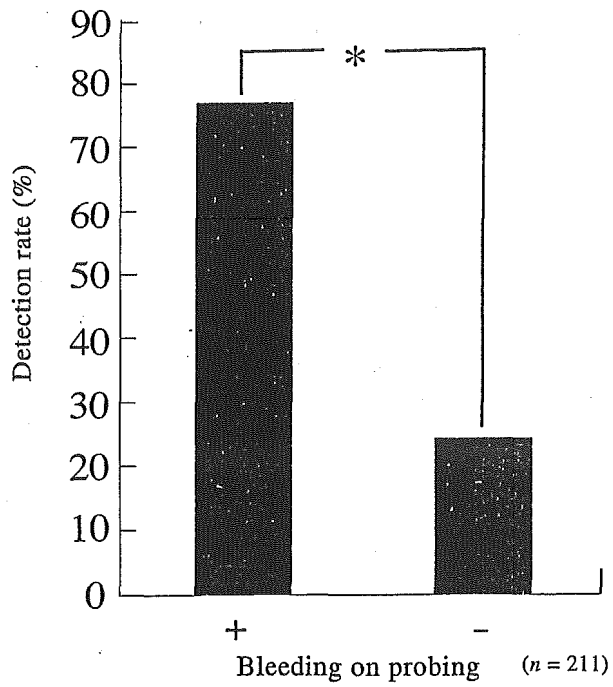

Fig. 7. Relationship between BOP and detection of *C. rectus*. A significant difference was seen between the BOP positive and negative groups. *$P < 0.001$ by chi-squared test.

As a result that relationship between PD and the detection rate of *C. rectus* using dot-blot analysis was statistically significant; it was 21.7% in the PD ≤ 2, 59.3% in the 3 ≤ PD ≤ 5 group, and 96.5% in the PD ≥ 6 group. As periodontal pockets became deeper, the detection rate of *C. rectus* increased. *Campylobacter rectus* was almost always detected in the deeper periodontal pockets, showing a relationship between *C. rectus* and the degree of progression of periodontitis. This finding was similar to the detection rate of *C. rectus* in adult periodontitis studied with DNA probe by Lippke *et al.* (35).

In the BOP positive group, the detection rate was 76.5%, and in the BOP negative group, it was 24.2%; this difference was also significant ($P < 0.001$). This result demonstrated that there is a relationship between the level of periodontal inflammation and the presence of *C. rectus*.

By dot-blot analysis, the detection rates were 21.1% and 26.4% in the GI: 0 and GI: 1 groups, respectively. In the GI: 2 and GI: 3 groups, they were 77.9% and 88.5%. Between the GI: 0 and GI: 1 groups, and between the GI: 2 and GI: 3 groups, the differences were not significant, but they were significant between GI: 0 and GI: 2 and between GI: 0 and GI: 3. Thus *C. rectus* was often detected in large numbers in severe inflammation sites. Seida (26), using fluoroimmunoassay, reported that there were relationships between the detection rate of *C. rectus* BOP and GI. Rams *et al.* (9), using the culture method, reported that *C. rectus* increased in periodontally active sites. Papapanou *et al.* (36) also detected *C. rectus* in 81.0% of patients by the culture method. Söder *et al.* (37) found a detection rate of 65.7% in localized periodontitis sites and discussed its pathogenic significance.

The advantage of our dot-blot analysis with the prepared monoclonal antibody against the S-layer is that it makes it possible to examine many clinical plaque samples at one time. This detection method is also economical, simple, and convenient. Detection is possible in 2.5 h. Thus, the dot-blot analysis can be done in conditions close to chairside without having to prepare special equipment. Using this bacterial detection method, it is possible for the patient to receive information in the same day. Further improvements are necessary, including a possibility of detecting major periodontopathogens such as *P. gingivalis*. *Campylobacter rectus* colonies were easily separated from a blood agar plate by colony-blot. and the combination of colony-blot and culture method gives greater advantage for isolation and identification of *C. rectus*

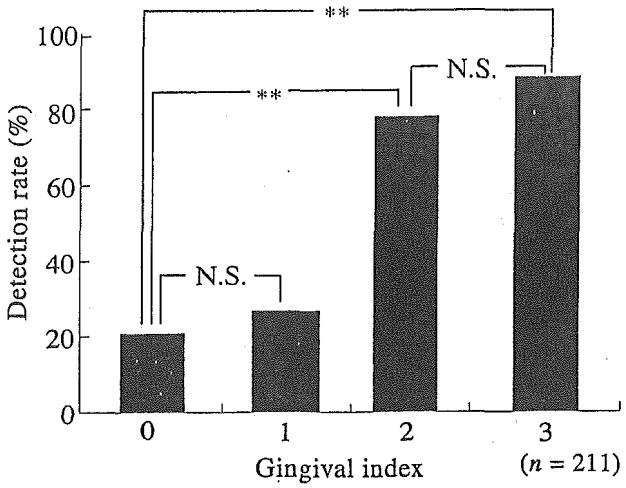

Fig. 8. Relationship between GI and detection of *C. rectus*. In the GI: 0 and GI: 1 groups, the detection rates were low and not significantly different. However, between GI: 0 and GI: 2 and between GI: 0 and GI: 3, significant differences were statistically recognized. $^{**}P < 0.001$; N.S.: no significance by chi-squared test.

compared with conventional culture method, which requires many steps of analysis. These results indicated that the dot-blot system using CRT-2 is useful tool to analyze a role of *C. rectus* in etiology of periodontitis.

Acknowledgements

We thank Dr B. L. S. Pierce, a professional editor who is the Life Science Coordinator for the Asian Division of the University of Maryland University College, for critical reading of this manuscript. We thank Dr S. Kokeguchi for providing *C. rectus* ATCC 33238 S-layer (–) strain.

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Ecological and Immunopathological Implications of Oral Bacteria in *Helicobacter pylori*-Infected Disease

Katsuji Okuda,* Ryuta Kimizuka,* Akira Katakura,* Taneaki Nakagawa,* and Kazuyuki Ishihara*

Increasing evidence has linked colonization by *Helicobacter pylori* with the development of gastritis and peptic ulcer disease. *H. pylori* resides primarily in the gastric mucosa without invading the gastric epithelium, causing persistent mild gastric inflammation. There are many reports examining the relationship between colonization by microorganisms in the stomach and oral cavity. We found that some oral bacteria are able to trap *H. pylori* cells, but oral bacteria inhibit *H. pylori* growth in vitro. In cases where *H. pylori* was detected in oral cavity samples, including oral cancer surface samples, we suggested that this species had colonized the stomach and were present in the oral cavity only as a transient organism. We demonstrated that periodontopathic *Campylobacter rectus* strains posses proteinaceous antigens, including heat shock proteins that share antigenicity with antigens of *H. pylori* strains. These cross-reactive antigens between *H. pylori* and *C. rectus* may be related to the induction of immunopathological responses in periodontal tissues and the stomach. We concluded that *H. pylori* could not survive in the human oral cavity; however, there would be an interrelationship between periodontal disease due to *C. rectus* and stomach diseases due to *H. pylori*. *J Periodontol 2003;74:123-128.*

KEY WORDS

***Campylobacter rectus*; *Helicobacter pylori*; immune response; oral cavity/microbiology; periodontal diseases/microbiology; stomach diseases/microbiology.**

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Helicobacter pylori infection is a risk factor for peptic and duodenal ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer.[1] The detection of this microorganism in the oral cavity has been reported by several groups,[2-10] who demonstrated the microorganism in dental plaque and saliva, which would implicate the oral cavity as a potential reservoir for *H. pylori* or a possible route of transmission to other sites. However, other studies reported no detection of *H. pylori* from dental plaque samples.[11-15] The present study focused on the role of *H. pylori* detected by polymerase chain reaction (PCR) of oral cavity samples in the transmission and ecological interactions with oral endogenous bacterial species.

H. pylori is reported to elicit antibody production.[16] Some research has shown that the salivary IgG level can reflect colonization by *H. pylori* and suggested that a saliva sample is useful for the diagnosis of *H. pylori* infections.[17,18] Bogstedt et al.[19] suggested that salivary IgA increases in *H. pylori*-infected patients, but the clinical value of IgA antibodies in *H. pylori*-related gastritis remains controversial. In addition, cross-reactivity has been reported between *H. pylori* and other bacteria.[20] Various kinds of bacterial species have been observed in the oral cavity, and they continuously stimulate the host immune system. In periodontal lesions, the numbers of *Campylobacter rectus*, a periodontopathic bacterium, increase with the development of periodontitis.[21] During a study of antibody

responses to *H. pylori* strains, we found antibodies that were cross-reactive between *H. pylori* and *C. rectus*.[22] In the present article, we discuss the immunopathological role of the cross-reacted antigens in periodontal disease and *H. pylori*-related stomach disease.

SOME ORAL BACTERIA COULD TRAP *H. PYLORI*

It is well known that various kinds of microorganisms form biofilms in which organisms are intimately associated with each other and the solid substratum through binding and inclusion within an expolymer matrix; such systems are widely distributed in nature and disease.[23,24] In the oral cavity, multi-bacterial species form biofilms, which are well-ordered structures on dental plaque, the tongue, and other oral soft tissue surfaces.[25] In forming a community of bacterial species in oral biofilms such as dental plaque, coaggregation activity is a significant factor.[26]

We found that strains of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* strongly coaggregated with *H. pylori* strains[27] (Fig. 1). We demonstrated that heating *P. gingivalis* and *F. nucleatum* cells abolished coaggregation with *H. pylori* strains, but the heating of *H. pylori* cells did not. Coaggregation between *P. gingivalis* and *H. pylori* was inhibited by the addition of arginine or lysine, and coaggregation with *F. nucleatum* was inhibited by the addition of EDTA. But the addition of sugar substrata did not affect their coaggregation reactions. These results suggest that *P. gingivalis* and *F. nucleatum* cells trap *H. pylori* cells. However, some salivary components may inhibit trapping by these periodontal disease-associated bacteria. Mentis et al.[28] reported that crude mucin preparations derived from the saliva of 20 different donors inhibited the adherence of *H. pylori* strains to erythrocytes. In addition, they showed that treatment with neuraminidase resulted in a substantial reduction of the inhibitory activity of salivary mucin.

To determine whether the *H. pylori* detected in oral cavity samples by PCR is derived from the stomach, we compared the restriction polymorphism of the PCR products of various *H. pylori* strains.[29] After treating the respective PCR products with various restriction enzymes, restriction polymorphisms were compared. Since we could not identify any differences in the restriction fragment length polymorphisms of the PCR products examined in this study, *H. pylori* may have originated from the stomach by vomiting or reflux and then been trapped by some oral bacteria.

ANTAGONISTIC ACTIONS OF ORAL BACTERIA AGAINST *H. PYLORI*

We found that various oral bacterial species inhibited the growth of *H. pylori* strains in stab-cultures,[27,29] as shown in Table 1. Samples extracted from culture supernatants of *Streptococcus mutans* and *Prevotella intermedia* showed strong growth inhibitory activity against *H. pylori*

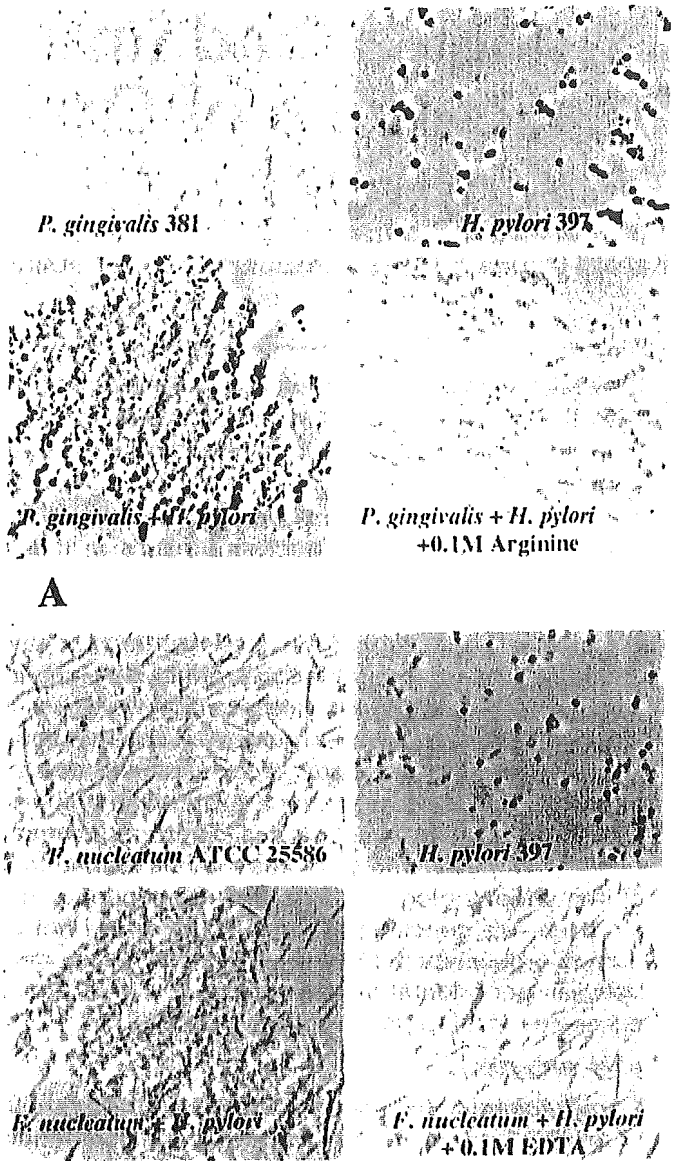

Figure 1.
Coaggregation between oral bacterial species and H. pylori *strains.* **A.** *Coaggregation between* P. gingivalis *and* H. pylori. **B.** *Coaggregation between* F. nucleatum *and* H. pylori.

strains. The growth inhibitory activities of most oral bac teria were adversely affected by heating at 80°C fc 60 minutes or by protease treatment, indicating tha these bacteria produce bacteriocin-like inhibitory prc teins against *H. pylori* strains.

The morphological characteristics of *H. pylori* strair cultured with culture supernatants of *S. mutans* (*P. intermedia* were examined using phase contra microscopy. Coccal cell numbers of *H. pylori* culture in the oral bacterial culture supernatant were signi cantly higher than those in the control.[29] It is possib that the morphological changes caused by oral bact

Table 1.

Growth Inhibition of a Clinical *Helicobacter pylori* Strain by Oral Bacterial Species

Oral Bacterial Strain	Clinical *H. pylori* Strain
Streptococcus sanguis ATCC 10556	+
Streptococcus oralis ATCC 10557	++
Streptococcus mitis ATCC 9811	++
Streptococcus salivarius ATCC 9758	+
Streptococcus mutans Ingbritt	++
Streptococcus sobrinus 6715	++
Actinomyces naeslundii ATCC 12104	++
Porphyromonas gingivalis ATCC 33277	+
Prevotella intermedia ATCC 25611	++
Prevotella nigrescens ATCC 33563	++
Fusobacterium nucleatum ATCC 25586	+
Actinobacillua actinomycetemcomitans ATCC 43718	+

+: Inhibitory radius of <5 mm; ++: inhibitory radius of 5 mm.

rial action result in a failure to isolate *H. pylori* from the oral cavity. The inhibition of *H. pylori* growth by oral bacterial products may affect the viability of the microorganism in the oral cavity.

H. PYLORI MAY HAVE ONLY A TRANSIENT PRESENCE IN THE ORAL CAVITY

Correa et al. indicated that colonization of *H. pylori* in the stomach is a risk factor for gastric cancer.[30] However, little is known about the role of *H. pylori* in oral cancer. To clarify whether *H. pylori* can be detected in oral cancer lesions, we examined colonization by the culture method as well as reverse transcription PCR (RT-PCR).[29]

The oral cancers examined were squamous cell carcinomas and salivary gland adenocarcinoma, adenocryptic carcinoma, mucoepidermoid carcinoma, dysplasia, and leukoplakia.[29] A selective medium, described previously, for *H. pylori* that contained calf heart, brain extract, and antibiotics was also used to isolate the microorganism. Swab samples of the oral mucosa and cancer lesion surfaces were obtained before endoscopic examination and were subjected to RT-PCR and culturing. Gastric biopsy specimens and gastric aspirates were obtained during endoscopy. The RT-PCR results showed that stomach samples from 54 of 116 patients with peptic ulcer or gastritis were positive for *H. pylori* (Table 2). We detected *H. pylori* by RT-PCR in 12 dental plaque samples from 54 patients with gastric infections of *H. pylori*. In contrast, there were only 2 positive swabs from patients with peptic ulcer or gastritis in which there were no *H. pylori* detected. In these 2 patients, only the oral samples were positive; it is possible that we failed to detect *H. pylori* present in their gastric samples. We detected *H. pylori* by RT-PCR in 11 of 21 swab samples of oral cancer patients whose stomach samples were positive. However, there were no positive RT-PCR results obtained from swab samples from 37 oral cancer patients whose stomach samples were negative. We isolated *H. pylori* strains in gastric samples from 29 of 54 patients (53.7%) whose RT-PCR were positive, but none from RT-PCR negative patients or oral samples. We can conclude that colonization and growth of this microorganism are difficult in the human oral cavity.

CROSS REACTED ANTIGENS BETWEEN *C. RECTUS* AND *H. PYLORI*

Karvar et al.[31] reported that evaluating serum IgG against *H. pylori* is a useful tool for the detection of *H. pylori*, but that evaluation of IgA against *H. pylori* is not. During a study of antibody responses to *H. pylori* strains,[22] we found an antibody that was cross-reactive with *C. rectus*. Figure 2 shows a scattered plot of anti-*H. pylori* ATCC 43504 and *C. rectus* ATCC 33238 antibody titers measured by enzyme linked-immunosorbent assay (ELISA). The salivary IgA antibody levels against *H. pylori* correlated well with those against *C. rectus* ($P < 0.0002$, $r = 0.907$), and the saliva IgG levels against *H. pylori* also correlated with those against *C. rectus* ($P < 0.0001$, $r = 0.891$). These results indicated that the local IgA production was elicited by an antigenic stimulus from either *C. rectus* or *H. pylori*.

Immunoblotting analyses using rabbit anti-*C. rectus* serum against sonicated antigens of *C. rectus* and *H. pylori* strains are shown in Figure 3. Sonicated antigens from not only *C. rectus* but also *H. pylori* formed many clear bands. These bands were produced by all *H. pylori* tested in this study. We confirmed that rabbit antiserum against *H. pylori* cells reacted to sonicated

Table 2.

Detection of *H. pylori* by RT-PCR

	Stomach Samples		Oral Swab Samples	
	Positive	Negative	Positive	Negative
Patients with peptic ulcer or gastritis	54	62	12	42
			2	60
Patients with oral cancer	21	37	11	10
			0	37

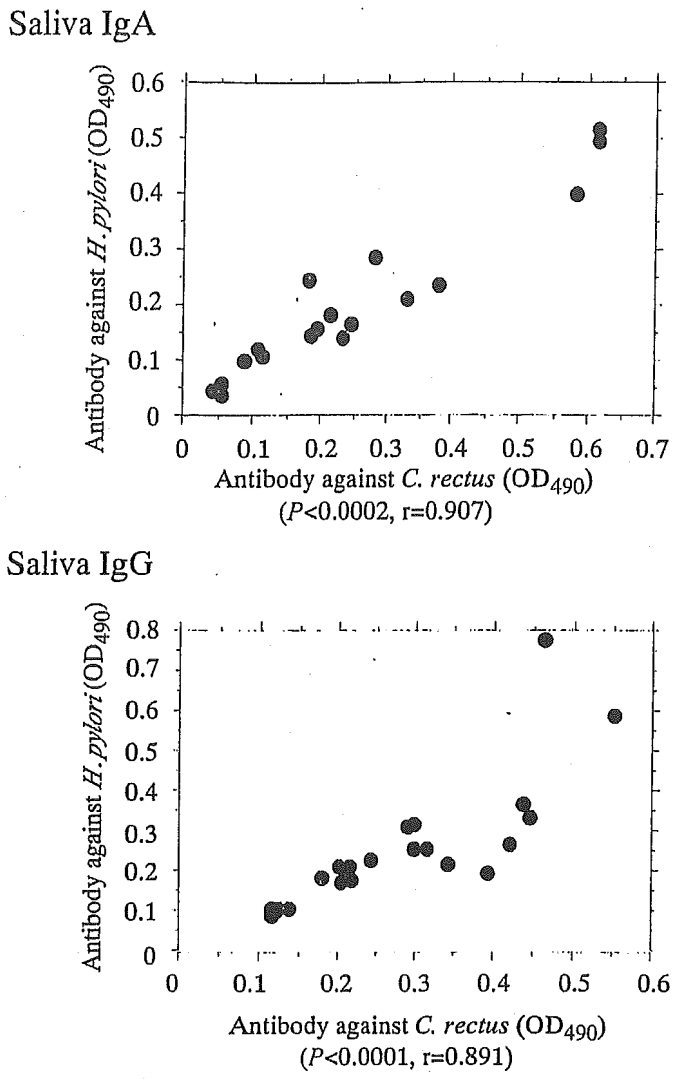

Figure 2.
Correlations between salivary antibody levels against H. pylori *and those against* C. rectus.

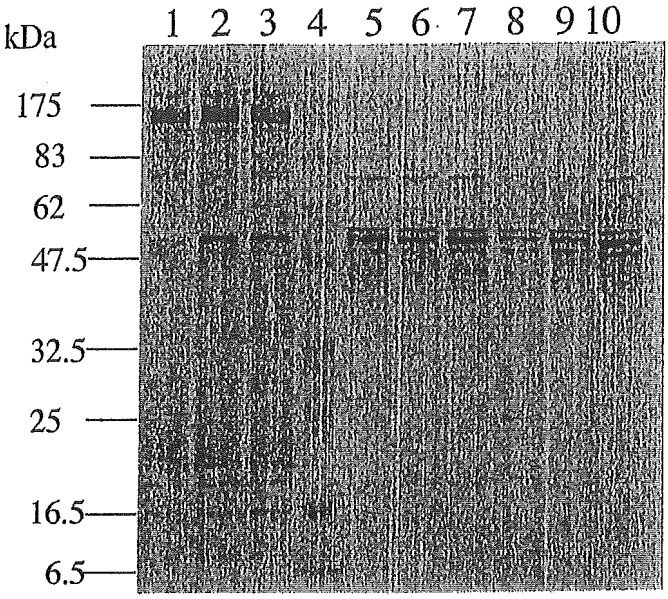

Figure 3.
Cross-reactivity of sonicated antigens extracted from H. pylori *and* C. rectus *strains. The sonicated antigens reacted with antiserum against whole cells of* C. rectus *strain. Lanes 1 to 3 are sonicated antigens of clinical strains of* C. rectus. *Lanes 5 to 10 are sonicated antigens of clinical strains of* H. pylori. *Lane 4 is the molecular size standard.*

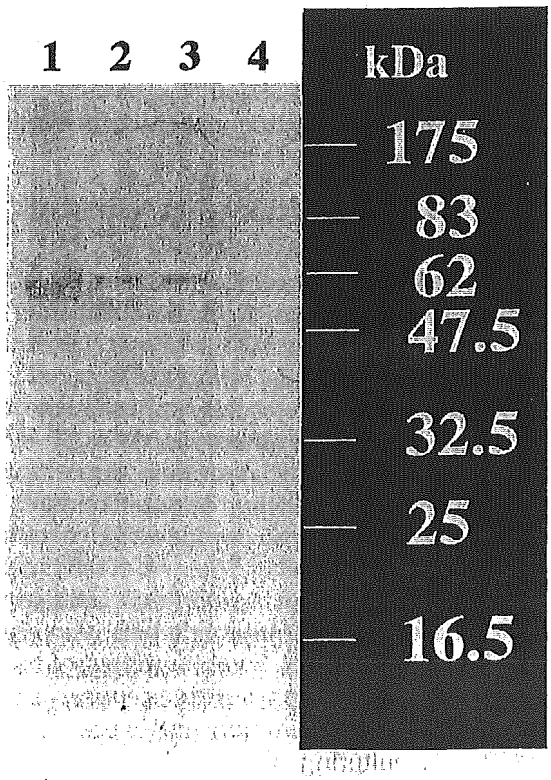

Figure 4.
Reactivity of sonicated antigens from H. pylori *and* C. rectus *strain with monoclonal antibody against* H. pylori *HSP 60. Lane 1 is a sonicated antigen from a clinical strain of* H. pylori *Kobe 856. Lanes 2 and 3 are sonicated antigens from strains of* C. rectus *CCUG 19168 and ATCC 3323, respectively. Lane 4 is molecular size standard.*

antigens from both *H. pylori* and *C. rectus* strains. After proteinase K treatment of the sonicate, these bands disappeared in both species, suggesting that the antigenically similar protein antigens exist in strains of *H. pylori* and *C. rectus*. We found that absorbing human serum from an *H. pylori*-infected subject with *C. rectus* cells resulted in the disappearance of some strongly reactive bands. This result demonstrates the cross-reactive antibody production in humans.

We found that the monoclonal antibody against heat shock protein (HSP) 60 of *H. pylori* reacted with sonicated antigens from *C. rectus* strains,[22] as shown in Figure 4. The 60 kDa cross-reactive HSP is equivalent to GroEL of *Pseudomonas aeruginosa* HSP.[32] Many oral bacterial species have HSPs that share antigenicity with host HSPs.[33,34] Hinode et al.[35] reported an immunoreactive GroEL-like protein in *C. rectus* strains. The 68 kDa protein in *C. rectus* observed in the present